Cloning and expression of a unique inorganic pyrophosphatase from *Bacillus subtilis*: evidence for a new family of enzymes

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Abstract **An open reading frame located in the COTF-TETB intergenic region of *Bacillus subtilis* was cloned and expressed in *Escherichia coli* and shown to encode inorganic pyrophosphatase (PPase). The isolated enzyme is Mn^{2+}-activated, like the authentic PPase isolated from *B. subtilis*. Although 13 functionally important active site residues are conserved in all 31 soluble PPase sequences so far identified, only two of them are conserved in *B. subtilis* PPase. This suggests that *B. subtilis* PPase represents a new family of soluble PPases (a Bs family), putative members of which were found in *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Streptococcus mutans* and *Streptococcus gordonii*.**

Key words: Pyrophosphatase; Exopolyphosphatase; Polymerase chain reaction; Recombinant enzyme; Manganese; *Bacillus subtilis*

1. Introduction

Inorganic pyrophosphatase (EC 3.6.1.1) catalyzes specifically the hydrolysis of pyrophosphate to orthophosphate. This reaction provides a thermodynamic pull for many biosynthetic reactions [1–3] and is essential for life [4–6]. PPases require bivalent metal ions for catalysis, with Mg^{2+} conferring the highest activity. The best-studied PPases are those from *Escherichia coli* and *Saccharomyces cerevisiae*, which have been extensively characterized by X-ray crystallography [7–10] and site-directed mutagenesis in combination with kinetic and thermodynamic measurements [11–17]. All known soluble PPases are homologous proteins, whose active site structure is evolutionarily very well conserved [18,19].

In the 1960s, Tono and Kornberg [20] carried out initial characterization of soluble PPase from *Bacillus subtilis*, and recently the enzyme was characterized in more detail [21,22]. In contrast to other soluble PPases, *B. subtilis* PPase is activated by preincubation with Mn^{2+} ions, which presumably affect the equilibrium between the active trimer and the less active dimer [22]. In the present study, the gene encoding *B. subtilis* PPase was cloned and expressed in *E. coli*, the enzyme was compared in terms of amino acid sequence with other soluble PPases and shown to be a first member of a new, Bs family of soluble PPases.

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Abbreviations: PPase, inorganic pyrophosphatase; P_i, orthophosphate

2. Materials and methods

Restriction endonucleases, T4 DNA polymerase, Taq polymerase and a DNA ligation kit were purchased from Takara Shuzo, [α-^{32}P]dCTP (3000 Ci/mmol) and a DNA sequencing kit were from Amersham, a Gene Clean II kit was from Bio 101, plasmid vector pET-3a was from Novagen, marker proteins for SDS-PAGE were from Bio-Rad, phenyl-Sepharose CL-4B was from Pharmacia and DEAE-HPLC column was from Tosoh.

B. subtilis strain AC327 was used throughout this study. *E. coli* JM109 [23] and *E. coli* BL21(DE3) (Novagen) were used as hosts in the cloning and expression, respectively. The strains were grown in LB broth or on 2×YT plates [23]. Ampicillin (50 μg/ml) was added when required.

B. subtilis PPase was expressed in *E. coli* under the inducible phage T7 promoter by making use of the pET system (Novagen). The open reading frame encoding *B. subtilis* PPase was amplified by polymerase chain reaction (PCR) using a 5′-sense oligonucleotide primer containing a restriction site for *Nde*I (5′-GCGCATATGGAAAAGATACTTATTTTCG-3′, the restriction site is underlined) and a 3′-reverse complement primer with a *Bam*HI site (5′-AAGGATCCTTATTCAGCCATTGCGTCT-3′). The PCR product was digested with *Nde*I and *Bam*HI, ligated into the vector pET-3a (Novagen), cut with the same restriction enzymes and transformed into *E. coli* JM109 and *E. coli* BL21(DE3) for DNA sequencing [24] and expression, respectively. The expression was induced for 3–6 h by 0.5 mM IPTG.

The authentic PPase was purified from *B. subtilis* as described previously [21], whereas the recombinant enzyme was purified to homogeneity from *E. coli* by a simplified procedure including only phenyl-Sepharose CL-4B column chromatography and DEAE-HPLC [21]. About 26 mg of pure enzyme was obtained from 12 g of cell paste with a yield of 45%. To stabilize the enzyme, 1.5 mM $MnCl_2$ was included in buffers at all purification steps. The purified enzyme gave a single band on SDS-PAGE gels stained with Coomassie brilliant blue [25]. The N-terminal sequence of the authentic enzyme was determined by an automatic Edman degradation method with an Applied Biosystem 477A/120A gas-phase sequenator.

PPase activity was assayed at 37°C as described previously [26]. The assay medium contained 1 mM PP_i, 2 mM $MgCl_2$ and 20 mM Tris-HCl buffer (pH 7.3). One unit of activity corresponds to 1 μmol of PP_i converted per minute. Protein concentration was determined with a Pierce BCA protein assay kit, with BSA as standard.

3. Results and discussion

Soluble PPase isolated from *B. subtilis* was purified to homogeneity and its N-terminal residues 1–39 were determined by automatic Edman degradation method giving the sequence: 1-M-E-K-I-L-I-F-G-H-Q-N-P-D-T-D-T-I-X-S-A-I-A-Y-A-D-L-K-N-K-L-G-F-N-A-E-P-V-R-L-39, where X denotes an unidentified residue. By searching the Swiss-Prot database, this sequence was found to be identical with an N-terminal gene-deduced sequence of a hypothetical 34-kDa protein encoded by a non-identified open reading frame located in the COTF-TETB intergenic region of *B. subtilis* [27].

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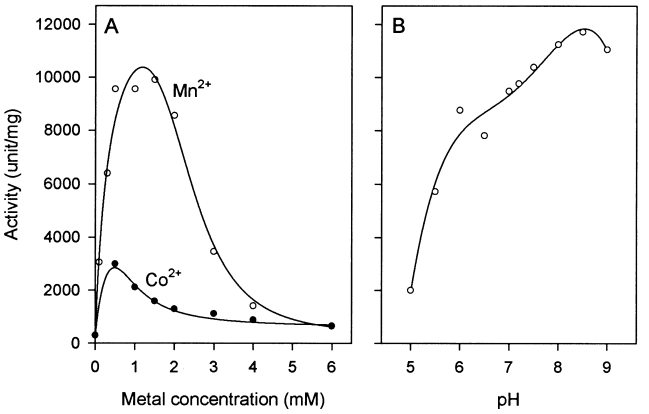

Fig. 1. Activation of *B. subtilis* PPase by Mn^{2+} and Co^{2+}. A: Metal ion dependence of the activation. The enzyme (0.1 mg/ml), dialyzed against 20 mM Tris-HCl, pH 7.3, containing 0.1 mM EDTA, was incubated for 5 min in the same buffer containing different concentrations of $MnCl_2$ (○) or $CoCl_2$ (●). Enzyme activities were measured immediately after a 500–1000-fold dilution of the samples with the metal containing buffer. B: Dependence of enzyme activation in the presence of 1.5 mM Mn^{2+} on pH. The incubation conditions were same as for A. The buffers used were 20 mM MES/NaOH (pH 5.0–7.0) and Tris-HCl (pH 7.2–9.0).

This open reading frame was amplified by PCR and its sequence was verified to be identical to that reported previously [27]. By its size, the hypothetical protein is similar to *B. subtilis* PPase (34–36 kDa [22]).

The PCR product was expressed under T7 promoter in *E. coli*, yielding transformants with 180-fold higher PPase activity than the host strain, clearly indicating that the open reading frame encodes a PPase. Like authentic *B. subtilis* PPase [22], the recombinant enzyme purified to homogeneity was activated by preincubation with Mn^{2+} and Co^{2+} (Fig. 1A), but not with Mg^{2+}, Ca^{2+}, Sr^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+} or Ni^{2+}. Full activation was obtained in 2 min of incubation. The activation was strongly dependent on metal ion concentration (Fig. 1A) and pH (Fig. 1B). Maximal activation was observed at pH 8.5 with 1.5 mM Mn^{2+} (Fig. 1). At pH > 9, manganese hydroxide precipitated, limiting our ability to study the activation at high pH values. The specific activity of the enzyme at pH 7.3 was 323 and 9900 U/mg before and after the activation by 1.5 mM Mn^{2+}, corresponding to the k_{cat} values of 180 and 5500 s^{-1}, respectively. Interestingly, the former k_{cat} value is about the same as those for *E. coli* and yeast PPases [11,12], whereas the latter is greater by an order of magnitude.

According to its amino acid sequence, *B. subtilis* PPase is unique among known soluble PPases. There are 31 soluble PPase sequences currently available in the GenBank: prokaryotic PPases have 164–233 amino acid residues per subunit, whereas eukaryotic PPases have 211–310 residues per subunit. In this respect, *B. subtilis* PPase, with 309 amino acid residues per subunit, resembles eukaryotic PPases. Furthermore, *B. subtilis* PPase shows little sequence similarity to other soluble PPases, typical examples of which are shown in Fig. 2. Sequence identity between PPase of *B. subtilis* and *E. coli* is as low as 17%, whereas internal identity of the 21 available prokaryotic PPase sequences varies from 31% (*Haemophilus influenzae* vs. *E. coli*) to 61% (*Legionella pneumophila* vs. *E. coli*). Fan rom evolutionary point of view, it is interesting to note that *Bacillus stearothermophilus*, a close relative of *B. subtilis*, has a PPase which is very similar to other soluble PPases [28], but completely different from *B. subtilis* PPase (Fig. 2): identities of *B. stearothermophilus* PPase vs. *E. coli* PPase and *B. subtilis* PPase are 44 and 15%, respectively.

An even more striking difference is observed when comparing active site residues, which are evolutionarily very well conserved in other soluble PPases [18,19]. In 31 known PPase sequences, 13 functionally important active site residues are conserved in all sequences, but only two of them (D70 and D97 in *E. coli* PPase numbering) are conserved in *B. subtilis* PPase (Fig. 2).

In addition to soluble PPases, plants and certain bacteria have a membrane-bound PPase, which works as a reversible proton pump. Membrane-bound PPases differ in many respects from soluble PPases – they are much larger (660–

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                          20                  40                  60                  80
Bsb-PPase:  MEKILIFGHQ NPDTDTICSA IAYADLKNKL GFNAEPVRLG QVNGETQYAL DYFKQESPRL VETAANEVNG VILVDHNERQ
Bst-PPase:  .......... .......... .......... .......... .......... .......... .AFENKIVEA FIEIPTGSQN
E-PPase:    .......... .......... .......... .......... .......... ..SLLNVPAG KDLPEDIY.V VIEIPANADP
Y-PPase:    .......... .......... .....TYTTR QIGAKNTLEY KVYIEKDGKP VSAFHDIPLY ADKENNIFNM VVEIP.RWTN

                         100                 120                 140                 160
Bsb-PPase:  QSIKDIEEVQ VLEVIDHHRI ANFETAEPLY YRAEPVGCTA TILNKMYKEN NVKIEKEIAG LMLSAIISDS LLFKSPTCTD
Bst-PPase:  .KYEFDKE.. .........R GIF.KLDRVL YSPMFYPAEY GYLQNTLA.. .......... LDGDPL..DI LVITTNPTFP
E-PPase:    IKYEIDKE.. .........S GAL.FVDRFM STAMFYPCNY GYINHTLS.. .......... LDGDPV..DV LVPTPYPLQP
Y-PPase:    AKLEITKEET LNPIIQDTKK GKL.RFVRNC FPHHGYIHNY GAFPQTWEDP NVSHPETKAV GDNDPI..DV LEIGETIAYT

                         180                 200                 220                 240
Bsb-PPase:  QDVAAAKELA EIAGVDAEEY GLNMLKAGAD LSKKTVEELI SLDAKEFTLG SKKVEIAQVN TVDIEDVKKR QAELEAVISK
Bst-PPase:  GCVIDTRVIG YLNMVDSGEE DAKLI..GVP VED...PRFD EVRSIEDLPQ HKLKEIAHFF ER.YKDLQ.. .GKRTEIGTW
E-PPase:    GSVIRCRPVG VLKMTDEAGE DAKLV..AVP HSK.LSKEYD HIKDVNDLPE LLKAQIAHFF EH.YKDLEK. .GKWVKVEGW
Y-PPase:    GQVKQVKALG IMALLDEGET DWKVI..AID INDPLAPKLN DIEDVEKYFP GLLRATNEWF RI.YK.IPD. .GKPENQFAF

                         260                 280                 300
Bsb-PPase:  VVAEKNLDLF LLVITDILEN DSLALAIGNE AAKVEKAFNV TLENNTALLK GVVSRKKQVV PVLTDAMAE. .......... .
Bst-PPase:  EGPEAAAKLI DECIARYNEQ K......... .......... .......... .......... .......... .......... .
E-PPase:    ENAEAAKAEI VASFERAKNK .......... .......... .......... .......... .......... .......... .
Y-PPase:    SGEAKNKKYA LDIIKETHDS WKQLIAGKSS DSKGIDLTNV TLPDTPTYSK AASDAIPPAS LKADAPIDKS IDKWFFISGS V
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Fig. 2. Alignment of four PPase sequences. The sequences were aligned with PILEUP (gap weight and length weight values of 2.0 and 0.6, respectively) of the GCG package. Numbering is for the sequence of *B. subtilis* (Bsb) PPase. The other sequences shown are for soluble PPases from *B. stearothermophilus* (Bst), *E. coli* (E), and *S. cerevisiae* (Y). The 13 essential, active site residues which are conserved in all 31 complete soluble PPase sequences currently available in the GenBank are in bold face. Only two of these 13 residues are conserved in *B. subtilis* PPase (underlined).

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                                20                                  40                  60
Bsb-PPase:  .MEKILIFGH QNPDTDTICS AIAYADL... .........K NKLGFNAEPV RLGQVNGETQ YALDYFKQES PRLVETA.AN
      Smu:  .MSKILVFGH QNPDSDAIGS SYAFAYL... .........AR EAYGLDTEAV ALGEPNEETA FVLDYFGVAA PRVITSAKAE
      Sgo:  .MSKILVFGH QNPDSDAIGS SMAYAYL... .........KR Q.LGVDAQAV ALGNPNEETA FVLDYFGIQA PPVVKSAQAE
      Afu:  MEHVVYVVGH KNPDTDSVCS AIAFAYLWNK WKEGGNVAKM MKIEAEAKPV IQGDVNPETK YVLEKFGFEV PEIMTNGEG.
      Mja:  ...MRYVVGH KNPDTDSIAS AIVLAYFLDC Y.....PARL .......... ..GDINPETE FVLRKFGVME PELIESAKG.

                      80                  100                 120
Bsb-PPase:  EVNGVILVDH NERQQSIKDI EEVQVLEVID HHRIANFETA EPLYYRAEPV GCTATILNKM YKENNV.... ....KIEKEI
      Smu:  GAEQVILTDH NEFQQSVADI AEVEVYGVVD HHRVANFETA NPLYMRLEPV GSASSIVYRM FKEHSV.... ....AVSKEI
      Sgo:  GAKQVILTDH NEFQQSIADI REVEVVEVVD HHRVANFETA NPLYMRLEPV GSASSIVYRL YKENGV.... ....AIPKEI
      Afu:  ..KKVALVDH SEKAQTVDGI DKAEVVAIVD HHKIGDVTTP QPILFVNLPV GCTATVIKLL FDKTGV.... ....EIPKDI
      Mja:  ..KEIILVDH SEKSQSFDDL EEGKLIAIID HHKVG.LTTT EPILYYAKPV GSTATVIAEL YFKDAIDLIG GKKKELKPDL

          140                 160                 180                 200
Bsb-PPase:  AGLMLSAIIS DSLLFKSPTC TDQDVAAAKE LAEIAGV.DA EEYGLNMLKA GADLSKKTVE ELISLDAKEF TLGSKKVEIA
      Smu:  AGLMLSGLIS DTLLLKSPTT HPTDKAIAPE LAELAGV.NL EEYGLAMLKA GTNLASKSAE ELIDIDAKTF ELNGNNVRVA
      Sgo:  AGVMLSGLIS DTLLLKSPTT HASDPAVAED LAKIAGV.DL QEYGLAMLKA GTNLASKTAA QLVDIDAKTF ELNGSQVRVA
      Afu:  AGILLSSILS DTVIFKSATT TELDKEVAEE LAKIAGIDDL TKFGVEIKAK LSAVDDLTAM DIIKRDYKDF DMSGKKVGVG
      Mja:  AGLLLSAIIS DTVLFKSPTT TDLDKEMAKK LAEIAGISNI EEFGMEILKA KSVVGKLKPE EIINMDFKNF DFNGKKVGIG

          220                 240                 260                 280
Bsb-PPase:  QVNTVDIEDV KKRQAELEAV ISKVVAEKNL DLFLLVITDI LENDSLALAI GNEAAKVEKA FNVTLENNTA LLKGVVSRKK
      Smu:  QVNTVDIAEV LERQAEIEAA IEKAIADNGY SDFVLMITDI INSNSEILAI GSNMDKVEAA FNFVLENNHA FLAGAVSRKK
      Sgo:  QVNTVDINEV LERQNEIEEA IKASQAANGY SDFVLMITDI LNSNSEILAL GNNTDKVEAA FNFTLKNNHA FLAGAVSRKK
      Afu:  QIELVDLSLI ESRIDEIYEA MKKMKEEGGY AGIFLMLTDI MKEGTELLVV TDYPEVVEKA FGKKLEGKSV WLDGVMSRKK
      Mja:  QVEVIDVSEV ESKKEDIYKL LEEKLKNEGY DLIVFLITDI MKEGSEALVV GN.KEMFEKA FNVKVEGNSV FLEGVMSRKK

          300
Bsb-PPase:  QVVPVLTDAM AE.
      Smu:  QVVPQLTESF NA.
      Sgo:  QVVPQLTESF NG.
      Afu:  QVVPPLEKAF AEL
      Mja:  QVVPPLERAY NG.
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Fig. 3. Alignment of the sequences of *B. subtilis* PPase and its four homologues. Numbering is for the sequence of *B. subtilis* (Bsb) PPase. The other sequences shown are the putative PPases of the Bs family from *S. gordonii* (Sgo), *S. mutans* (Smu), *A. fulgidus* (Afu), and *M. jannaschii* (Mja). The residues conserved in all five sequences are underlined, whereas the conserved Asp, Glu, Arg and Lys are in bold face.

770 amino acid residues per monomer) and do not have any sequence similarity to soluble PPases [29–31]. The *B. subtilis* PPase described here is clearly a soluble PPase, and it does not have any sequence similarity to membrane-bound PPases.

A search through the GenBank indicated the following four proteins having the highest identity ($>30\%$) to the primary structure of *B. subtilis* PPase: 'hypothetical exopolyphosphatases' of *Streptococcus mutans*, *Methanococcus jannaschii*, *Archaeoglobus fulgidus* and intrageneric coaggregation-relevant adhesin protein of *Streptococcus gordonii*. These four proteins have 307–311 amino acids/subunit, which is very close to the value of 309 for *B. subtilis* PPase. Identities of these four proteins are 57, 44, 40 and 57%, respectively, vs. *B. subtilis* PPase and only 13, 16, 18 and 15%, respectively, vs. *E. coli* exopolyphosphatase [32]. This suggests that the four proteins mentioned above are in fact *B. subtilis* type PPases. This fits very well with the whole genome analysis showing that *B. subtilis* [33], *M. jannaschii* [34,35] and *A. fulgidus* [36] seem to have no typical *ppa* gene for the soluble PPase. On the other hand, *B. subtilis* PPase is clearly not an exopolyphosphatase, which is typically larger by size (396–513 amino acids/subunit) [32,37] and does not degrade PP_i at all [38], or degrades it at a very low rate [39].

The 13 functionally important conserved active site residues of the known soluble PPases include five Asp, two Glu, two Lys, one Arg and two Tyr residues (Fig. 2). Sequence alignment of *B. subtilis* PPase and its four homologues, putative members of a new family of PPases, showed nine Asp, three Glu, four Lys and one Arg residues which are conserved in all these five proteins (Fig. 3) – these are obvious candidates for the active site residues, if the same types of residues are responsible for catalysis in both families of soluble PPases. Furthermore, there are several conserved polar residues of other types (two Asn, three Gln, four His, four Thr and five Ser), which may also have functional roles (Fig. 3). Expression and characterization of these four hypothetical PPases will eventually provide a test for these predictions. There are also several highly conserved regions, the most prominent of which is an eight residue long sequence near the C-terminus (294-SRKKQVVP-301). A search through the protein sequence database has indicated that only the five proteins shown in Fig. 3 have a perfect match with this octapeptide, suggesting this sequence to be a fingerprint for the *B. subtilis* type of PPases. It will be interesting to see what is the role of this highly conserved region in PPase structure and functioning.

In summary, *B. subtilis* has a unique type of soluble PPase, which is completely different from the closely related PPases studied so far [18,19]. Accordingly, *B. subtilis* PPase is a first example of a new family of soluble PPases, which we name Bs family. Putative representatives of the Bs family are also observed in four other bacterial strains, at least two of which, like *B. subtilis*, do not seem to have a typical *ppa* gene in their genomes.

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